## APPENDIX A

Please replace the paragraph beginning at page 17, lines 10 through 23 with the following:

Figure 1 panel A shows the location of the two polymorphisms, called BSP-A1496G (SEQ ID NO. 13) and BPS-G1869A (SEQ ID NO. 14), in the bone sialoprotein gene promoter. Panel B shows the location of the polymorphism, called MGP-C242A (SEQ ID NO. 15), in the matrix gla protein gene promoter. Panel C shows the location of the polymorphisms, called OPN-G520A (SEQ ID NO. 16) and OPN-T1825C (SEQ ID NO. 17), in the osteopontin gene promoter. The wild type sequences encompassing the four polymorphic sites for all three said promoters are shown with the nucleotide at the polymorphic position in bold and with the substituting nucleotide - also in bold - positioned above the polymorphic site. All nucleotide numbering is relative to base pair number 1, which is the most 5' nucleotide of each of the promoter sequences as published in the GenBank nucleotide database.

Please replace the paragraphs bridging pages 22 through 24 beginning at page 22, line 21 through page 24, line 20 with the following:

DNA analyses. Screening for the BSP-A1496G (SEQ ID NO. 13) and the BSP-G1869A (SEQ ID NO. 14) polymorphisms (basepair numbering according to numbering of BSP promoter sequence submitted to GenBank, accession #L24756), the MGP-C242A (SEQ ID NO. 15) polymorphism (basepair numbering according to numbering of MGP promoter sequence submitted to GenBank, accession #M55270), as well as the OPN-G520A (SEQ ID NO. 16) and OPN-T1825C (SEQ ID NO. 17) polymorphisms (basepair numbering according

to numbering of osteopontin promoter sequence submitted to GenBank, accession #D14813) were performed as follows:

The polymerase chain reaction (PCR) was used to amplify approximately 250 bp long DNA fragments of the BSP, MGP, and OPN promoters encompassing the BSP-A1496G (SEQ ID NO. 13), BSP-G1869A (SEQ ID NO. 14), MGP-C242A (SEQ ID NO. 15), OPN-G520A (SEQ ID NO. 16), and OPN-T1825C (SEQ ID NO. 17) polymorphic basepairs. PCR techniques are well known in the art and it would be within the ambit of a person of ordinary skill in this art to identify primers for amplifying a suitable section of the BSP, MGP and OPN genes including the positions 1496bp and 1869pb in the BSP promoter, the position 242bp in the MGP promoter, and the positions 520bp and 1825bp in the osteopontin promoter. PCR techniques are described for example in patents US4683202 or EP0200362B1. Two hundred ng of genomic DNA was added to 25 μl reaction containing 1x Taq polymerase buffer with 12.5 mM $MgCl_2$ (Perkin Elmer), 5 nmol of each dNTP, 20 pmol of forward and reverse primer, and 1.25 units of AmpliTaq Gold (Perkin Elmer). The reaction was heated to 95°C for 9 minutes followed by 35 cycles of 95°C for 30 seconds, 46°C (BSP-A1496G and BSP-G1869A polymorphisms) or 49°C (MGP-C242A polymorphism) or 46°C (OPN-G520A polymorphism) or 48°C (OPN-T1825C) for 30 seconds and 72°C for 30 seconds - the latter incubation with a 5 second time extension per cycle. The reaction was finally incubated 7 minutes at 72°C for completion of the extension reaction. Primer sequences for PCR amplification of DNA fragments encompassing the BSP-A1496G, BSP-G1869A, MGP-C242A, and OPN-G520A and OPN-T1825C polymorphic basepairs were:

BSP-1496G polymorphism primer set:

Forward primer: 5' - GAA AAG ATA TAT ATA GAA GCC CAA G - 3' (SEQ ID No. 1)

Reverse primer: 5' - TAA TAT CAT TTG ATG TTT CCT CCT G - 3' (SEQ ID No. 2)

BSP-G1869A polymorphism primer set:

Forward primer: 5' - TTC TTT CGA CAT AGT GAA AAC ACG T - 3' (SEQ ID No. 3)

Reverse primer: 5' - CGT GGA TTC TCA CCA GAA AAC - 3' (SEQ ID No. 4)

MGP-C242A polymorphism primer set:

Forward primer: 5' - CAG TGA GAA AGC TCA TCA CTT GGT C - 3' (SEQ ID No. 5)

Reverse primer: 5' - ATT CTC CCA TCC ATC CAT CCA TGC A - 3' (SEQ ID No. 6)

OPN-G520A polymorphism primer set:

Forward primer: 5' - CGC TGG AAT TAA GAA AAT TGG TAG A - 3' (SEQ ID No. 7)

Reverse primer: 5' - GTT GTC AAT TTA GTG GAG GGA GAT C - 3' (SEQ ID No. 8)

OPN-T1825C polymorphism primer set:

Forward primer: 5' - GAG TAG TAA AGG ACA GAG GCG AGC T - 3' (SEQ ID No. 9)

Reverse primer: 5' - CTA GCT TTT TCA TTT ACG GGA TGG G - 3' (SEQ ID No. 10)

Please replace the paragraph beginning at page 27, lines 15 through 27 with the following:

<u>Results</u>

Five previously unknown polymorphisms were identified by sequencing specific promoter regions from the human BSP gene promoter, the human MGP gene promoter, and the human OPN gene promoter following a PCR amplification of 40 DNA samples from healthy women. The BSP-A1496G <u>(SEQ ID NO. 13)</u>, BSP-G1869A <u>(SEQ ID NO. 14)</u>, MGP-C242A <u>(SEQ ID NO. 15)</u>, OPN-G520A <u>(SEQ ID NO. 16)</u> and OPN-T1825C <u>(SEQ ID NO. 17)</u> polymorphisms were coded as Xx, Yy, Zz, Bb, and Ss, respectively, where the uppercase letter signifies presence of the wild type base pair at the given polymorphic

position and the lowercase letter signifies presence of the base pair different from the wild type base pair at the given polymorphic position.

Please replace the paragraph bridging pages 27 and 28 beginning at page 27, lines 30 through page 28, line 9 with the following:

Table 1 shows the genotype distribution of DNA samples from the 18 years study for all identified polymorphic sites: BSP-A1496G (SEQ ID NO. 13), BSP-G1869A (SEQ ID NO. 14), MGP-C242A (SEQ ID NO. 15), OPN-G520A (SEQ ID NO. 16) and OPN-T1825C (SEQ ID NO. 17). The left panel shows the actual number of samples categorized into three genotypes for the 3 identified polymorphic sites. The right panel displays the same analysis as the left except that the numbers represent the percent of total samples analyzed for each polymorphic site.

wt = XX, YY, ZZ, BB or SS

hz = Xx, Yy, Zz, Bb or Ss

pm = xx, yy, zz, bb or ss

Please replace the paragraph beginning at page 29, lines 3 through 20 with the following:

The genotype distributions for the 5 polymorphisms are shown in table 1. For the BSP-A1496G (SEQ ID NO. 13) polymorphism the homozygous wild type genotype was the most abundant, followed by the heterozygous and homozygous polymorphic genotypes, with the homozygous polymorphic groups being quite small. In the case of BSP-G1869A (SEQ ID NO. 14) polymorphism, the wild type genotype, as defined by the BSP gene promoter sequence from GenBank, was rare, the heterozygous genotype was 10 times more frequent

and the homozygous polymorphic genotype was twice as frequent as the heterozygous. For the MGP-C242 (SEQ ID NO. 15) polymorphism the heterozygous genotype was the most abundant followed by the homozygous wild type and homozygous polymorphic genotypes. In the case of the OPN-G520A (SEQ ID NO. 16) polymorphism, the homozygous polymorphic genotype was the most abundant followed by the heterozygous and the wild type homozygous genotypes. The genotype distribution of the OPN-T1825C (SEQ ID NO. 17) polymorphism was, generally, the same as for the MGP-C242A (SEQ ID NO. 15) polymorphism.

Please replace the paragraphs beginning at page 32, lines 1 through page 34, line 16 with the following:

From the table it is clear that the BSP-A1496G (SEQ ID NO. 13) and BSP-G1869A (SEQ ID NO. 14) polymorphic sites, especially when combined, are good sites for predicting whether an individual is genetically predisposed for high or low BMC/BMD. The OPN-T1825C (SEQ ID NO. 17) polymorphism only has a marginal influence on BMC/BMD on its own. However, when the OPN-T1825C (SEQ ID NO. 17) polymorphism is combined with the BSP-G1869A (SEQ ID NO. 14) polymorphism the percent separation of genotypes is better than either polymorphism alone (Table 2). On the other hand, the MGP-C242A (SEQ ID NO. 15) and OPN-G520A (SEQ ID NO. 16) polymorphisms are, at first glance, not suitable sites for such a prediction. None of the identified polymorphisms appeared to have a statistically significant impact on the change in bone mass over time (data not shown). Age, height and weight of the individuals involved in the 18 years study did not differ significantly between any of the genotype groups (data not shown).

These observations strongly indicate that the BSP polymorphisms influence peak bone mass rather than the rate of bone loss. To substantiate this an analysis of the variation of BMD as measured 4 times on the same individual from 1977 to 1995 for the different genotypes was performed. In 1977 the average age of the individuals included in the 18 years study was 51.1 years, thus ending at 69.1 years in 1995. The expected outcome of a plot of the means of BMD for one of the two BSP polymorphisms as a function of time would be two parallel curves, each representing BMDs measured in individuals with the wild type genotype and BMDs measured in individuals with the polymorphic phenotype. Figures 2 and 3 show that this is, indeed, the case for the BSP-A1496G (SEQ ID NO. 13) and BSP-G1869A (SEQ ID NO. 14) polymorphic sites. Moreover, the two BSP promoter polymorphism act in concert on peak bone mass to augment the mean BMD difference between genotypes even more than the isolated contributions of each polymorphism (Figure 4).

The role - if any - of the MGP-C242A (SEQ ID NO. 15) and OPN-G520A (SEQ ID NO. 16) polymorphisms in the MGP and OPN promoters on bone turnover was less clear from the first analyses compiled in table 2. However, when BMC values grouped according to genotype were plotted as a function of time a set of curves appeared suggesting that both the MGP-C242A (SEQ ID NO. 15) (Figure 5) and OPN-G520A (SEQ ID NO. 16) (Figure 6) polymorphic sites are determinants of rate of bone loss. It is especially noteworthy that the ZZ and Zz+zz curves as well as the BB+Bb and bb curves separate between 1979 and 1989, corresponding to an average age of 53.1 years and 63.1 years, indicative of a genetic phenomenon associated with the menopause. Like the BSP polymorphisms, the combined action of the MGP-C242A (SEQ ID NO. 15) and OPN-G520A (SEQ ID NO. 16) polymorphisms also leads to a bigger difference between genotypes than either would create alone (Figure 7).

According to the results compiled in table 2, the impact of the OPN-T1825C (SEQ ID NO. 17) polymorphism on BMC/BMD was only visible after it was combined with the BSP-G1869A (SEQ ID NO. 14) polymorphism. From a graph of BMC values grouped according to genotype and plotted as a function of time it is difficult to tell whether this polymorphism has an impact on rate of bone loss or peak bone mass, due to the proximity of the curves (Figure 8). However, combining this polymorphism with the BSP-G1869A (SEQ ID NO. 14) polymorphism gave rise to a set of time course curves clearly showing that these two polymorphisms cooperate in an additive fashion, and, hence, that the OPN-T1825C (SEQ ID NO. 17) polymorphism may influence peak bone mass, as the BSP-G1869A (SEQ ID NO. 14) polymorphism, rather than the rate of bone loss (Figure 9).

Finally, the association between genotype and urinary osteocalcin (N-MID®), Osteometer Biotech A/S) as well as urinary collagen type 1 C-terminal crosslinks (CrossLaps® Osteometer Biotech A/S) were examined. As expected there was no significant difference between the mean value of either of the biochemical bone turnover markers for the BSP-A1496G (SEQ ID NO. 13) and BSP-G1869A (SEQ ID NO. 14) polymorphic sites (data not shown). Also, no significant difference was observed between the mean value of the biochemical bone turnover markers for the MGP-C242A (SEQ ID NO. 15) and OPN-G520A (SEQ ID NO. 16) polymorphic sites. This is likely due to the equally paced bone loss of the ZZ and Zz+zz genotype groups as well as the BB+Bb and bb genotypes groups at the time of N-MID® and CrossLaps® measurement (1995) according to Figures 5 and 6.

Please replace the paragraph beginning at page 37, lines 1 through 19 with the following:

The impact of the identified polymorphic site on bone mass as represented by bone mineral content (BMC) and bone mineral density (BMD) measurements at the distal arm in 1977 and 1995, respectively, was analysed (Table 3). The percent difference between the genotype groups did not change significantly from 1977 to 1995, which implied that the OPG-A163G polymorphism exerted an influence on peak bone mass. The two polymorphisms, called BSP-A1496G (SEQ ID NO. 13) and BSP-G1869A (SEQ ID NO. 14), described above also have an impact on peak bone mass. Hence, it was of interest to examine whether any co-operation between the OPG polymorphism and either of the BSP polymorphisms existed. The combinations OPG-A163G/BSP-A1496G and OPG-A163G/BSP-G1869A showed that those [polymor-phisms] polymorphisms certainly act in a co-operative fashion, in that the t-test p-values for the null-hypothesis (i.e. no difference between the genotype groups) dropped to statistically significant values and the percent difference in mean BMC/BMD values for two genotype groups increased (Table 3).

Please replace the paragraphs beginning at page 38, lines 12 through page 39, line 19 with the following:

Thus, it is clear that the OPG-A163G polymorphism, especially in combination with the BSP-A1496G (SEQ ID NO. 13) and BSP-G1869A (SEQ ID NO. 14) polymorphisms, is a good site for predicting whether an individual is genetically predisposed for high or low BMC/BMD. Age, height and weight of the individuals involved in the 18 year study did not differ significantly between any of the genotype groups (data not shown).

To substantiate the initial indication, that the OPG/OCIF polymorphism influences peak bone mass, an analysis of the variation of BMC as measured 4 times on the same individual from 1977 to 1995 for the different genotypes was performed. In 1977 the

average age of the individuals included in the 18 year study was 51.1 years, thus ending at 69.1 years in 1995. The expressed outcome of a plot of the BMC means for the OPG-A163G polymorphism as a function of time would be two parallel curves, each representing BMCs measured in individuals with the wild type genotype and BMCs measured in individuals with the heterozygous or polymorphic homozygous phenotypes. Figure 11 shows that this is certainly the case. Moreover, the combination of the OPG-A163G and the BSP-A1496G (SEQ ID NO. 13) polymorphisms show that they act in concert on peak bone mass to augment the mean BMD difference between genotypes even more than the isolated contribution of each polymorphism (Figure 12). In fact, this co-operation is completely additive (Table 4), indicating that the two polymorphisms act on bone mass independently of one another. The numbers for the BSP-A1496G (SEQ ID NO. 13) and BSP-G1869A (SEQ ID NO. 14) polymorphisms in Table 4 are from the results presented in Figures 2 and 3. Also, the combination of the OPG-A163G and BSP-G1869A polymorphisms indicates a positive co-operation (Figure 13), which is almost additive (Table 4).

## APPENDIX B

Pending claims

Please amend the claims as follows:

4. (Once Amended) A method as claimed in Claim 3, wherein said allelic variation of the bone sialoprotein gene promoter is BSP-A1496G (SEQ ID NO. 13) or BSP-G1869A (SEQ ID NO 14).

5. (Once Amended) A method as claimed in Claim 3, wherein said allelic variation of the matrix gla protein gene promoter is MGP-C242A (SEQ ID NO. 15).

6. (Once Amended) A method as claimed in Claim 3, wherein said allelic variation of the osteopontin gene promoter is OPN-G520A (SEQ ID NO. 16) or OPN-T1825C (SEQ ID NO. 17).

9. (Once Amended) A method as claimed in Claim 8, wherein said allelic variation of the bone sialoprotein gene promoter is BSP-A1496G (SEQ ID NO. 13) or BSP-G1869A (SEQ ID NO. 14).